IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

APPLERA CORPORATION, MDS INC., and APPLIED BIOSYSTEMS/MDS SCIEX INSTRUMENTS, *Plaintiffs,* v. THERMO ELECTRON CORPORATION, *Defendant.*	C.A. No. 04-1230-GMS
THERMO FINNIGAN LLC, *Plaintiff,* v. APPLERA CORPORATION, MDS INC., and APPLIED BIOSYSTEMS/MDS SCIEX INSTRUMENTS, *Defendants.*	C.A. No. 05-110-GMS

APPENDIX TO AB/SCIEX'S ANSWERING CLAIM CONSTRUCTION BRIEF

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SPECIAL FEATURE:
TUTORIAL

An introduction to quadrupole–time-of-flight mass spectrometry

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A brief introduction is presented to the basic principles and application of a quadrupole–time-of-flight (TOF) tandem mass spectrometer. The main features of reflecting TOF instruments with orthogonal injection of ions are discussed. Their operation and performance are compared with those of triple quadrupoles with electrospray ionization and matrix-assisted laser desorption/ionization (MALDI) TOF mass spectrometers. Examples and recommendations are provided for all major operational modes: mass spectrometry (MS) and tandem MS (MS/MS), precursor ion scans and studies of non-covalent complexes. Basic algorithms for liquid chromatography/MS/MS automation are discussed and illustrated by two applications. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Although first introduced commercially only 6 years ago, quadrupole–time-of-flight (TOF) mass spectrometers have rapidly been embraced by the analytical community as powerful and robust instruments with unique capabilities. In particular, they combine the high performance of time-of-flight analysis in both the mass spectrometry (MS) and tandem MS (MS/MS) modes, with the well accepted and widely used techniques of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). While originally targeted at the analysis of peptides,[1,2] they are now applied to problems which range from nanospray analysis of biological samples to liquid chromatography (LC)/MS/MS of pharmaceutical preparations at high flow-rates. Their rapid acceptance is due to the attractive combination of high sensitivity and high mass accuracy for both precursor and product ions, and also to the simplicity of operation for those already familiar with LC/MS analysis on quadrupole and triple quadrupole instruments.

In this paper we will use the relatively generic term QqTOF to refer both to the technique and to the instrument, where Q refers to a mass-resolving quadrupole, q refers to an r.f.-only quadrupole or hexapole collision cell and TOF refers to a time-of-flight mass spectrometer. Historically, the development of the QqTOF followed closely after the development of the so-called ESI-TOF technique

(electrospray ionization time-of-flight). This technique uses the principle of orthogonal injection from a high-pressure ion source, and its history (and principles) are well described in several recent reviews.[3–5] The configuration can be regarded either as the addition of a mass-resolving quadrupole and collision cell to an ESI-TOF, or as the replacement of the third quadrupole (Q3) in a triple quadrupole by a TOF mass spectrometer. From either viewpoint, the benefits that accrue are high sensitivity, mass resolution and mass accuracy of the resulting tandem mass spectrometer in both precursor (MS) and product ion (MS/MS) modes.[1,2] Particular advantage for full-scan sensitivity (over a wide mass range) is provided in both modes by the parallel detection feature available in TOFMS. This advantage does not inherently apply to the more specialized modes analogous to the precursor ion, neutral loss, and 'MRM' (multiple reaction monitoring) scans of triple quadrupole systems, and until recently these scan methods could not be performed with any reasonable efficiency. However, new techniques are emerging to address this limitation, broadening the range of analytical problems to which this instrument can be applied. (Note that the term 'scan' cannot be applied to a TOF instrument, since it is not a scanning device; nevertheless, common terminology dictates the use of the term in certain situations). In addition, while the instrument was initially considered to be suitable only for qualitative analysis, it is clear that nothing prevents its use for quantitative applications, once the issues that affect the dynamic range are understood. To date, the best absolute sensitivity for targeted compounds (requiring the measurement

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of only a few types of ions) is still achieved with a triple quadrupole system; however, the increased specificity provided by the higher resolution QqTOF may provide S/N (signal-to-noise ratio) benefit in some analytical situations.

The popularity of the QqTOF has been significantly advanced by the rapid growth of semi-automated instrument control and data processing (also a major factor in the commercial success of the quadrupole ion trap), and by continuing improvements in the core performance characteristics of mass resolution and sensitivity. In addition, the recent development of a matrix-assisted laser desorption/ionization (MALDI) ion source for the QqTOF has provided new capabilities in MS and MS/MS, which expand the applications in biological research. The recent explosion in the field of protein research (including commercial proteomics) also appears to be contributing significantly to its commercial success. It therefore seems timely to provide a snapshot of the technology as it currently exists, describing the operating principles as they relate to important performance features such as sensitivity, mass resolution and mass accuracy, with illustrations from some of the major fields of application. We will try to outline, in as practical a fashion as possible, the important aspects of tuning and operation, the strengths and the limitations of the technique (perhaps debunking some common perceptions while supporting others), the advantages provided by the ion sources that are most widely used and the power of instrument control and data processing software. It should be noted that the last aspect is a moving target; it is likely that by the time this paper is read, new automation capabilities will have been developed and

exploited, and far more capabilities will have been proposed for the future.

BASIC PRINCIPLES OF OPERATION

As mentioned already, the QqTOF tandem mass spectrometer can be described in the simplest way as a triple quadrupole with the last quadrupole section replaced by a TOF analyzer. The properties of the triple quadrupole instrument have been treated thoroughly by Yost and Boyd.[6] In the usual QqTOF configuration, an additional r.f. quadrupole Q0 is added to provide collisional damping, so the instrument (Fig. 1) consists of three quadrupoles, Q0, Q1 and Q2, followed by a reflecting TOF mass analyzer with orthogonal injection of ions[2] (in one of the commercial instruments, the quadrupoles Q0 and Q2 are replaced by hexapoles;[1] however, the basic operating principles are the same).

For single MS (or TOFMS) measurements, the mass filter Q1 is operated in the r.f.-only mode so that it serves merely as a transmission element, while the TOF analyzer is used to record spectra. The resulting spectra benefit from the high resolution and mass accuracy of the TOF instruments, and also from their ability to record all ions in parallel, without scanning. In principle, it is still possible to perform Q1 scans for single MS analysis by using the TOF section as a total ion current detector only. However, owing to the described advantages of the TOF spectra, this operational mode is used for Q1 calibration and tuning only.

Ions are sampled from a high-pressure electrospray or APCI ion source through an r.f. ion guide Q0 into Q1. This additional quadrupole Q0 is used for collisional cooling and
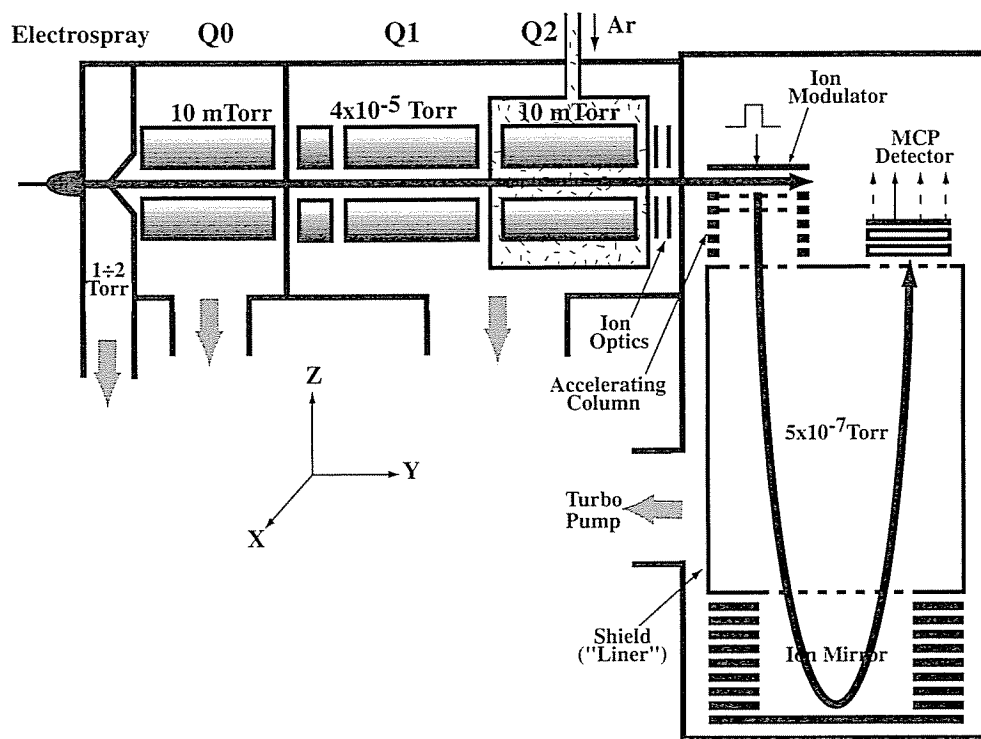

Figure 1. Schematic diagram of the tandem QqTOF mass spectrometer.

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focusing of the ions entering the instrument. Both Q0 and Q2 are operated in the r.f.-only mode: the r.f.-field creates a potential well that provides radial confinement of the precursor and/or fragment ions. Since the r.f. quadrupoles are normally operated at a pressure of several millitorr, they provide both radial and axial collisional damping of ion motion. The ions are thermalized in collisions with neutral gas molecules, reducing both the energy spread and the beam diameter and resulting in better transmission into and through both the quadrupole[7] and TOF[8] analyzers. After leaving the r.f. quadrupoles, ions are re-accelerated in the axial direction to the necessary energies with near-thermal energy spreads.

For MS/MS, Q1 is operated in the mass filter mode to transmit only the parent ion of interest, typically selecting a mass window from 1 to 3 thomson (Th) wide depending on the desire to transmit the full isotopic cluster. The ion is then accelerated to an energy of between 20 and 200 eV before it enters the collision cell Q2, where it undergoes collision-induced dissociation (CID) after the first few collisions with neutral gas molecules (usually argon or nitrogen). The resulting fragment ions (in addition to the remaining parent ions) are collisionally cooled and focused as described above. This step is even more important in QqTOF instruments than it is in triple quadrupoles because the TOF analyzer is much more sensitive to the 'quality' of the incoming ion beam than is Q3 in a triple quadrupole instrument, as will be discussed below.

Single mass spectra can be acquired either with or without collision gas in Q2. In the former case, all parameters in the quadrupole section are set as for MS/MS, but the collision energy is kept below 10 eV to avoid fragmentation. Both sensitivity and resolution benefit from the additional collisional focusing in the pressurized collision cell.

After leaving the collision cell, ions are re-accelerated to the required energy (usually several tens of electronvolts (eV) per unit charge), and focused by ion optics into a parallel beam that continuously enters the ion modulator of the TOF analyzer. Initially the modulator region is field-free, so ions continue to move in their original direction in the gap. A pulsed electric field is applied at a frequency of several kilohertz (kHz) across the modulator gap, pushing ions in a direction orthogonal to their original trajectory into the accelerating column, where they acquire their final energy of several keV per charge. From the accelerating column, ions arrive in the field-free drift space, where TOF mass separation occurs. The ratio of velocities (or energies) in the two orthogonal directions is selected such that ions reach the ion mirror and then the TOF detector naturally, without requiring an additional deflection in the drift region, which could affect the mass resolution.[9,10] A single-stage ion mirror provides compensation for the initial energy and spatial spread of the ions: ions originating at different vertical positions in the extraction gap of the modulator are focused on to a horizontal plane at the detector entrance. The detector is made of two microchannel plates in a chevron configuration. The electrostatic mirror and the ion detector are similar to those used with pulsed ionization sources (e.g. MALDI), apart from differences associated with a larger beam size (usually a few centimeters in

the y-direction, determined by the modulator aperture). Another major difference results from the requirement that the quadrupole section should be kept at low, near ground voltages. Therefore, positive ions are accelerated from ground to high negative voltages in the TOF section of the QqTOF instrument, rather than from high positive voltages to ground as in MALDI-TOF mass spectrometers. This 'inverted' configuration requires an extra shield within the TOF chamber to create a field-free drift region floated at high potential, but this shield must be designed carefully, since even a small penetration of an external field can seriously damage performance of the TOF.

All mass spectra from the TOF spectrometer (in both MS and MS/MS modes) are recorded with a time-to-digital converter (TDC). The TDC utilizes an ion counting technique with all its intrinsic advantages and drawbacks, which will be discussed in a separate section. A multiple anode detector combined with a multichannel TDC can be used to improve the dynamic range of the ion-counting technique.

Quadrupole transmission window

When used as a mass analyzer, quadrupoles operate with both r.f. and d.c. voltage components applied to the rods, and the reduced Mathieu parameters q_M and a_M are used to characterize the amplitudes of both components:[11]

$$q_M = \frac{4eV}{(m/z)\omega^2 r_0^2}; \qquad a_M = \frac{8eU}{(m/z)\omega^2 r_0^2} \qquad (1)$$

where e is the charge of an electron, V and ω are the amplitude and angular frequency of the r.f. voltage, respectively, U is the value of the d.c. voltage and r_0 is the inscribed radius of the quadrupole. Under normal (mass-analyzing) operational conditions, only ions within a narrow m/z window are transmitted corresponding to $q_M = 0.706$ and $a_M = 0.237$. In the r.f.-only operational mode ($a_M = 0$), quadrupoles serve as high-mass filters: ions with m/z values below a certain cut-off value corresponding to $q_M = 0.908$ are rejected.

Although there is no such sharp cut-off on the other end of the spectrum, transmission of ions with high m/z values suffers because of poorer focusing: the depth of the effective potential well is inversely proportional to m/z.[12] As a result, ions can be lost in any of the three multipoles on their way to the TOF section. In a single-MS mode with Q0, Q1 and Q2 in the r.f.-only mode, ions covering approximately an order of magnitude in m/z range are transmitted simultaneously into the TOF instrument. If a wider m/z range is required, the r.f.-voltage is modulated (stepped or ramped between two or more r.f. levels) during the spectrum acquisition, providing a larger transmission window averaged over time. However, it should be noted that the wider m/z range is obtained at the expense of the duty cycle: all light ions are lost when the r.f. is 'high,' and heavy ions are poorly focused in a shallow potential well when the r.f. is 'low.'

As mentioned above, either quadrupoles or hexapoles are used as an ion guide Q0 and collision cell Q2 in different versions of the QqTOF. Quadrupoles provide better ion focusing to the axis of the ion guide. On the other hand, hexapoles (or octapoles) have a steeper potential well close to the rods and are better in keeping the beam off the rods.

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Therefore, comparison of the performance of these devices is not straightforward, and goes beyond the scope of this paper. The above-mentioned transmission limitations are in effect for both types of multipoles,[12] and modulation of the r.f. voltage is necessary in both versions of QqTOF when a wide m/z range has to be covered.

Duty cycle and discrimination

In scanning mass analyzers, only one type of ion with particular m/z value is recorded at any given time, thus bringing their duty cycle below 0.1% when a full spectrum is recorded. Moreover, duty cycle is inversely proportional to mass resolution, because a narrower transmission window and a smaller step-size are required for higher resolution. In TOF analyzers with orthogonal injection, all ions are recorded in parallel (quasi-simultaneously), and the duty cycle is significantly larger than in scanning instruments. However, it is still far from 100%, and there is a mass discrimination, as discussed below.

To avoid spectral overlap, an injection pulse cannot be applied until the slowest ion from the previous pulse has reached the detector. For instruments without deflection, the velocity component v_y of an ion in the flight tube is the same as its velocity in the modulator region. Thus the slowest ions entering the modulator would spread out over a distance D by the time the next injection pulse is applied, if the region were long enough, where D is the distance between the modulator and detector centers. However, only a finite slice of this beam of length Δl can be accelerated and detected, where Δl is determined by the size of the apertures in the detector and/or the modulator. This sets an upper limit on the duty cycle for this m/z value as $\Delta l/D$.

If the incoming beam is monoenergetic, v_y is proportional to $1/\sqrt{m/z}$, so lighter ions would spread out over a distance larger than D between TOF pulses. Thus the duty cycle is proportional to $\sqrt{m/z}$ and there is a systematic discrimination against low-m/z ions:

$$\text{duty cycle } (m/z) = \frac{\Delta l}{D}\sqrt{\frac{m/z}{(m/z)_{max}}} \tag{2}$$

where $(m/z)_{max}$ corresponds to that with a flight time equal to 1/(TOF pulse frequency). Nevertheless, this discrimination is predictable, and signals at low m/z are normally stronger than at high m/z, so in most cases it is preferable to the decrease in transmission at high m/z which is characteristic of quadrupole mass filters. On the other hand, it is worth emphasizing that there is no further discrimination in TOF after the ion modulator, since ions with different m/z values move along the same trajectories as defined by voltages.

In most TOF instruments with orthogonal injection, the duty cycle is between 5% and 30%, depending on the m/z of the ion and the instrumental parameters. Whitehouse et al.[13] demonstrated an improvement in the duty cycle by trapping ions in a two-dimensional ion trap (an r.f. octapole with electrostatic apertures at each end), and then gating them in short bursts into the TOF spectrometer. With this method, 100% duty cycle was obtained over an m/z range of several hundred, but ions outside this range were lost partially or completely. In spite of the reduced m/z range, this method

can be very useful when applied in certain MS/MS modes of operation, where only selected fragment ions have to be monitored: precursor ion scans, neutral loss scans and MRM mode.[14]

Mass resolution of TOF

One of the main advantages of QqTOF instruments over triple quadrupoles is the high mass resolution of TOF, typically around 10 000 ($m/\Delta m$, where Δm is the full peak width at half-maximum (FWHM)). As a result of this, interfering peaks of ions having the same nominal mass can be resolved partially or completely, the charge state of multiply charged ions can in many cases be determined from their isotopic spacing, and signal-to-noise ratio is improved owing to grouping of ions into narrower peaks (increasing the peak height). In general, the higher the resolution the better, if it does not compromise sensitivity. Various factors limiting the resolution of TOF instruments were extensively discussed by Cotter,[15] and for the particular case of orthogonal injection TOF mass spectrometers by Dodonov and co-workers.[9,16] Therefore, only a brief outline will be given here.

In TOFMS, resolution is calculated as

$$R_{FWHM} = \frac{m}{\Delta m} = \frac{t}{2\Delta t} \approx \frac{L_{eff}}{2\Delta z} \tag{3}$$

where m and t are mass and flight time of the ion, Δm and Δt are the peak widths measured at the 50% level on the mass and time scales, respectively, Δz is the thickness of an ion packet approaching the detector and L_{eff} is the effective length of the TOF analyzer which takes into account the longer residence time in the electrostatic mirror and accelerating column. Note that when R is determined from t, there is an 'unfortunate' factor of 2 reducing the resolution, which originates from the square root dependence of the flight time on mass:

$$t = \frac{L_{eff}}{\sqrt{2eU_{acc}}}\sqrt{m/z} \tag{4}$$

where U_{acc} is the full accelerating voltage in the TOF.

In general, resolution is limited by the initial spatial and velocity spreads of the ions in the z dimension, the stability of power supplies, mechanical precision, grid scattering and the quality of microchannel plates. Let us briefly go through the most important limitations.

1. Ions start their motion along the z-axis from a gap in the modulator that is several millimeters wide. If the original thickness of the ion packet remained unchanged during its flight towards the detector, resolution according to Eqn (3) would be limited by $R \approx L_{eff}/2\Delta z \approx 500$, where $L_{eff} = 2$ m and $\Delta z = 2$ mm were used for an estimate. In fact, the situation could be even worse, taking into account that ions start from different equipotential planes in the extraction gap, and therefore obtain different energies from the electric field. The resulting energy spread can be as high as several hundred electronvolts, or a significant fraction of the total energy. Fortunately, the electrostatic mirror can take care of that and correct for both spatial and the resulting energy spread and compress the ion packet

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by the time it approaches the detector.[17] More energetic ions move faster in the drift space, but penetrate deeper into the mirror, and as a result stay longer in it. A single-stage electrostatic mirror is capable of the first-order time focusing, which is good enough if the energy spread is less than 5% of the total energy.

2. Both long- and short-term stability of the power supplies is critical for any TOF analyzer. High-frequency voltage variations lead to peak widening and loss of resolution, while slow changes result in a mass shift and the necessity to recalibrate the instrument more often.

3. High resolution and large size of the ion packets in the y-dimension (a few centimeters) set very strict requirements for mechanical tolerances in QqTOF mass spectrometers. Most importantly, all electrodes (grids, rings, etc.) of the modulator, accelerator, ion mirror and detector should be parallel to each other and perpendicular to the TOF axis. To achieve a resolution of 10 000 with an analyzer 2–3 m long, mechanical tolerances should be in the micrometer range. Flatness of all grids is also critical.

4. Microchannel plate (MCP) detectors are detectors of choice for TOFMS in general and for QqTOF in particular, because they provide a flat conversion surface with a large area, suitable for recording ion packets that are large in the y-dimension and thin along the z-axis. However, the MCP may produce a significant time spread because of the different degrees of penetration of ions into microchannels. Therefore, MCPs with the smallest channel diameter should be used.

5. The effect of the initial velocity spread in the z-direction on resolution is probably the largest. Consider two ions of the same mass starting from the same plane in the modulator, but with opposite directions of the initial velocity. The ion moving in the 'wrong' direction will turn around in the repelling electric field of the extraction pulse and will get to the same starting plane with the same velocity as the other ion, thus becoming its indistinguishable twin. However, it will always be behind those ions which chose the 'right' direction initially. The associated delay is called the 'turn-around time,' and it may provide a significant input into the peak width Δt in Eqn (2). There are two usual ways to reduce the effect of turn-around time on resolution: to apply stronger electric fields in the extraction gap (to speed up turn-around), and to build longer analyzers (to increase the flight time). However, both methods have their limitations: a stronger field leads to a larger energy spread of ions that cannot be corrected for by the electrostatic mirror, and there are common sense limits to increasing the length of instruments.

An increase in the flight path, without increasing the physical dimensions by much, can be achieved by using additional electrostatic mirrors placed in different geometries.[18] Although multiple-reflection TOF analyzers are less compatible with over-sized ion packets produced by the orthogonal injection method, two recent papers have demonstrated their usefulness for improving resolution of ESI-TOF[19] and QqTOF[20] instruments. However, the higher resolution of these instruments is achieved at the expense

of other instrumental parameters such as m/z range[19] and sensitivity.[20]

Basics of tuning the instrument

This small section certainly cannot replace manufacturers' manuals, but a few words might be appropriate. Once tuned, the TOF section usually does not require any voltage adjustments for months. Since orthogonal TOF has more-or-less fixed operating characteristics, the only parameters that are changed from one experiment to another are the extraction pulse frequency and width, which depend on the required m/z range. In general, switching to an opposite ion polarity means reversing all voltages (except the detector bias voltage); however, some values might need to be changed to obtain the best resolution: amplitude of the extraction pulses and/or voltages in the ion optics which direct the ion beam into the TOF (Fig. 1).

Tuning the quadrupolar section is very similar to that of triple quadrupoles, with some differences resulting from a different element being grounded (skimmer, collision cell or the ion modulator). Most of the voltage differences applied to ion-optical electrodes can usually be fixed; only those parameters that provide extra flexibility are left under operator control: declustering voltage(s), collision energy, r.f. levels (for r.f.-only quadrupoles), the width of transmitted window (when Q1 is resolving) and parameters of the ion source. A higher collision gas pressure is usually required in QqTOF compared with triple quadrupoles for the reasons described above. Higher pressure, however, may lead to a longer residence time of ions in Q2 and formation of adducts as a result, so the use of a weak axial electric field driving ions out of collision cell may be beneficial.[21]

ION RECORDING AND DYNAMIC RANGE

Although the method of recording the ion signal might be considered a detail which does not need to be discussed in this type of paper, there are certain features of the detection system which can affect the qualitative and quantitative response, and which require elaboration. Two types of detection systems are used in TOF mass spectrometers—transient recorders (TRs) and time-to-digital converters (TDCs). Transient recorders are analog systems which digitize the ion current from the output of the MCP (microchannel plate) detector, and are mainly used in MALDI-TOF instruments because they have a wide dynamic range. However, they are not typically suited for single ion detection because of the inherent background noise associated with analog detection. ESI-TOF and QqTOF instruments, in contrast, use TDCs for data registration. This is primarily because the ion signal (or, in fact, peak amplitudes) from these instruments is much lower in magnitude than that from a MALDI-TOF system, especially in an MS/MS mode where detection of individual ion events is important in order to achieve a good signal-to-noise ratio. Each ion that strikes the MCP creates a pulse of electrons at the anode, which is ~1–2 ns in width, not much shorter than the actual width of a mass peak in high resolution instruments. The pulse is amplified, and the leading edge of the pulse (after discrimination) is used to trigger a

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timing pulse which is sent to the TDC. The TDC registers the arrival time of the pulse (relative to the start time of the TOF extraction pulse), and the event and arrival time are stored in memory. Each TOF acceleration pulse (occurring at a rate of a few kilohertz) results in a spectrum of arrival times, and these individual spectra are summed in memory over the course of the acquisition period (e.g. 1 s), forming a mass spectrum which is a histogram of the events from several thousand pulses. Typical ion fluxes range from a few ions per second up to a few hundred thousand ions per second at the detector; this corresponds to a range from less than one ion per TOF pulse up to a few hundred ions per TOF pulse, distributed across the mass range.

Another important difference between transient recorders and TDCs is that TRs are sensitive to the pulse shape produced by a single ion pulse at the detector: the MCP pulse width (1–2 ns) is convoluted into the ion peak width in the recorded mass spectrum, and therefore affects mass resolution. Since TDCs are normally used together with a 'constant fraction discriminator,' their performance (time resolution) is not affected by the MCP pulse width or its amplitude.

One of the important characteristics of any mass spectrometer system is the dynamic range, which is usually defined as the range of either ion counts or sample concentration over which a linear response is obtained. Transient recorder systems have a wide dynamic range for individual mass peaks, although both the MCP plates and the digitizer can be saturated by high ion counts in certain situations. The dynamic range of the TDC, on the other hand, is limited by the counting dead time—the period after each ion event when the TDC itself is unable to register another count. Dead times are typically several nanoseconds long, of the same order as the width of a mass peak, but usually less than the spacing between mass peaks which are 1 Th apart. In each TOF pulse, only one ion can be recorded during a dead time so that if two ions of the same m/z value arrive at the detector, only the first is recorded. The result is that intense mass peaks become distorted by depletion of the top and right side of the peak, so that the peak intensity

is suppressed and the centroid shifted to the left. Note the difference with respect to scanning instruments: in QqTOF both peak amplitude and mass accuracy (centroid) suffer owing to saturation effects. Since the ion arrival time is governed by Poisson statistics, saturation effects can be corrected by applying a correction factor to each time-measurement interval in the mass spectrum:

$$C_i' = \frac{C_i}{1 - \sum C_j'/f} \tag{5}$$

where C_i is the measured count rate (in ions per time bin per second) for time bin number "i", C_i' is the corrected (true) count rate for that bin, and f is the TOF pulsing frequency. Equation (5) is applied to each time bin in the spectra from left to right, and the summation of C_j is performed over the time bins corresponding to the dead time to the left of bin "i". This algorithm corrects both peak shape and centroid when applied across the spectrum, and can be used to extend the dynamic range by a factor of ~10-fold. An example is shown in Fig. 2, where the isotopic envelope of the 5+ charge state of bovine insulin (M_r 5736) is distorted by a high count rate. Applying the Poisson correction shown above restores the isotopic envelope to its true distribution.

Because the TOF records full mass spectra, another simple technique is to use smaller peaks in the spectrum to correct for intensity and mass shift errors. For example, second or third ^{13}C isotopic peaks can be used to extend the dynamic range (for singly charged ions), up until the point where the ion source response begins to saturate. Alternately, in the MS/MS mode a lower intensity fragment can be used to extend the dynamic range.

With the rapid progress in electronics, new TDCs are being developed with better time resolution (<0.3 ns) and dead time (a few nanoseconds). It is important, however, to understand that even a 'zero dead-time TDC' will still have the same main limitation: being an ion counting device, it cannot register more than one ion per time bin. One method of extending the dynamic range is by using multiple collection anodes, which are connected to independent channels
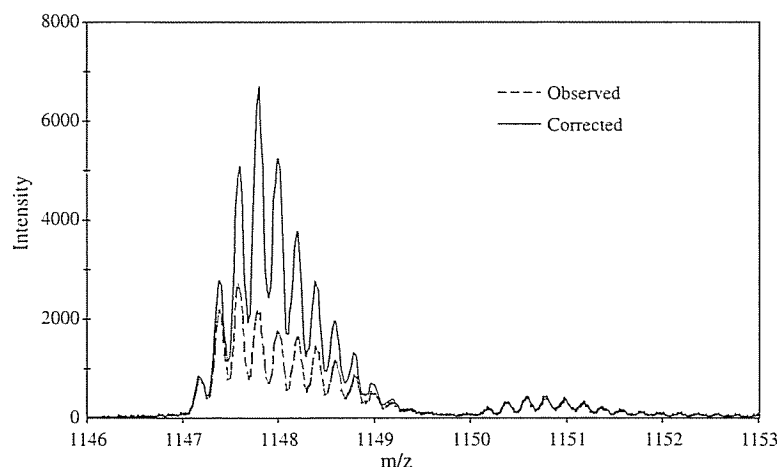

Figure 2. A segment of the ESI spectrum of bovine insulin. Peaks of the 5+ charge state are suppressed owing to the saturation of TDC (———— line); Poisson correction applied (- - - - line).

of a TDC.[22,23] Only a fraction of the ions strike each anode, so the count rate in any channel is reduced by the number of channels. Since the channels are independent, saturation does not occur until the count rate to individual channels (assuming the current is evenly divided) becomes saturated. Thus a four-anode detector and four-anode TDC can extend the dynamic range by a factor of up to fourfold. Because the ion current is not uniformly distributed across the anodes, however, practical gains are of the order of threefold.

It may be tempting to consider detuning the instrument in order to keep peak intensities in range, and to do it in a dynamic fashion. However, any simple method of reducing the ion intensity (detuning lenses, reducing detector voltage, changing the sprayer position or voltage) can also introduce mass or charge discrimination effects, as discussed in another section. If chromatography is employed, a simple technique is to take mass measurements on the sides of the eluting peak if the intensity is saturated at the top. This approach does not help for quantitation, where the complete chromatographic peak area must be taken. Methods such as those described above can readily be automated so that saturated peaks are detected by their absolute intensity and by their narrower peak shape, and then any of the above methods can be applied in software to correct the peak shapes and intensities.

MASS CALIBRATION AND MASS ACCURACY

While the QqTOF contains both a quadrupole and a TOF mass spectrometer, it is only the latter which is expected to give accurate mass. The quadrupole can usually be relied on to be stable over periods of from weeks to months once properly calibrated. The high mass accuracy of the TOF, which can be achieved in a very practical way, is due to two main factors: the high mass resolution and the simplicity (and hence predictability) of the mass calibration scale. The high mass resolution is important simply because it minimizes the possibility of overlap of two mass peaks, since the centroid of a peak can be significantly affected by even a minor underlying component of a slightly different m/z value. The accuracy and simplicity of the mass calibration is due to the inherently straightforward physics of the TOF mass spectrometer, in which $m/z \sim t^2 U_{acc}/L_{eff}^2$, as follows from Eqn (4), where U_{acc} is the acceleration voltage and L_{eff} is the length of the flight tube. As long as the points are not too close together, a simple two-point calibration is usually accurate over several hundred or even thousand thomson. This is in contrast to the situation with MALDI-TOF, where the flight time can be affected by ion source conditions such as surface distortion (sample morphology) and laser fluence, requiring a multipoint calibration curve (when delayed extraction is used) and internal standards for best mass accuracy. In the QqTOF, a mass accuracy of <5 ppm can be achieved as long as (i) there are no underlying interferences, (ii) there are sufficient ion statistics to measure the peak centroid accurately and (iii) the mass calibration function has been accurately measured. Factors (i) and (ii) are captured in an expression for the maximum achievable mass accuracy which was first suggested by Campbell and Hallyday:[24] accuracy $\sim 1/N^{1/2}R$ where N is the number of ions

counted in a mass peak and R is the mass resolution (higher resolution means a narrower and therefore taller peak, with more ions per time bin). Another important factor is that the resolution of time measurement must be sufficiently high to provide an accurate peak centroid. Experience has suggested that four time bins or more over the half-height peak width are sufficient to allow accuracy of <5 ppm to be achieved. Assuming that all peak shapes are the same, the centroid can be selected at any height (not just 50%); the narrower the selection for the centroid, the less likely it is that adjacent peaks will interfere, but fewer ions give input into the centroid calculation. A height of 50–25% usually provides a good compromise between a reasonable fraction of ions used and an attempt to avoid interferences.

Any drift in mass calibration is usually due to a temperature change, since both the power supply output and the length of the flight tube are a function of temperature. Typical TOF power supplies have ~ 25 ppm C^{-1} stability (+ or −) while stainless steel has a coefficient of expansion of about 18 ppm C^{-1}. A change in the voltage affects the mass scale proportionately, while a relative change in length has double the effect on the mass scale. Sometimes these can compensate for each other (if both drifts are in the same direction); however, normally any drift due to temperature change must be corrected for by mass calibration before or after a measurement. To provide semi-automatic correction, schemes involving continuous introduction of a calibration compound (multiplexed with the sample stream) have been employed.[25] While the ambient temperature can be measured and used to correct for a mass drift (assuming the instrument drift vs temperature has been well characterized beforehand), such a scheme is complicated by the problem that not all parts of the instrument have the same temperature response: the flight tube (liner) length, for example, changes much more slowly than the electronics because it is thermally insulated in the vacuum chamber. Any scheme that uses temperature compensation or correction must use measurement and control elements which are more precise and thermally stable than the instrument itself.

The form of the mass calibration function is $m/z = k(t - t_0)^2$, where t_0 represents any timing latency in the circuitry and k represents the instrumental parameters of V and L. Since t_0 is small and nearly independent of temperature, the effect of temperature can usually be corrected with a single point calibration which allows re-calculation of k. This conveniently allows tandem mass spectra to be accurately calibrated from the m/z of a single peak in the spectrum, if the accurate mass of the ion is known.

SINGLE MS OPERATION

In the single MS mode, all three quadrupoles serve as r.f.-only (non-resolving) ion guides, and mass analysis is performed in the TOF, as discussed earlier. TOF has the benefit of recording ions of all masses in parallel, but that does not mean that an unlimited m/z range (another advantage of TOF) can be monitored at no cost in sensitivity. There are two main reasons for this. First, the repetition rate of TOF extraction pulses has to be decreased to observe ions with higher m/z, otherwise peaks of heavy ions will overlap with

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light ions originating from the next extraction pulse. As a result, a wider m/z range will lead to a reduced duty cycle for light ions, as follows from Eqn (2).

Another factor that may reduce sensitivity when a wide m/z range is recorded is the necessity to step or ramp the r.f. voltage of quadrupoles, as described above. In one of the commercial QqTOF instruments, the decision to introduce the second step of r.f. can be made by software, based upon a simple criterion:

$$\frac{(m/z)_{max}}{(m/z)_{min}} > 5 \tag{6}$$

For example, if a TOF spectrum is recorded from m/z 200 to 900, all quadrupoles are run at the same constant r.f. level corresponding to low mass cut-off around m/z 150. However, if the mass range is extended to m/z 1200, voltages on the quadrupoles will be alternated between two r.f. levels in order to cover the whole m/z range. The newly introduced step will correspond to a cut-off of ~400 Th, and consequently ions with m/z from 200 to 400 will be recorded with a 50% efficiency compared with the previous case. Heavy ions (with $m/z > 900$) may also suffer due to poor confinement when the r.f. is at a 'low' level.

Although little can be done to eliminate these losses of ions, the general rule should be to avoid recording of unnecessary parts of spectra. It is worth noting that in contrast to quadrupoles, it is the ratio $(m/z)_{max}/(m/z)_{min}$ that is important, not the difference in these values. So, starting from m/z 50 or 100 may make a big difference. On the other hand, the percentage of time spent by quadrupoles at 'high' and 'low' r.f. levels can be varied so as to give a larger priority to the m/z range of particular interest.

In general, the sensitivity of QqTOF instruments in the single MS mode is high enough to produce a reasonable spectrum in 1 s or less, and this valuable feature is widely used in LC/MS applications. Although in most cases the desire is to record as many ions during a given time interval as possible, the ion counting technique of the TDC imposes strong limitations on scientists' dreams. This is especially true for the experiments performed with nanospray or nano-LC, where the ion current 'swallowed' into the mass spectrometer can be so high as to produce peak saturation beyond any software-correctable limits. At a first glance, the problem of 'too many ions' is easier to solve than the opposite one, and there are more ways to reject ions rather than to make them go through. It turns out, however, that most of these methods introduce some sort of unwanted discrimination based on either m/z value, intensity or molecular properties. One of the typical cases is illustrated by Plate 1, which shows peaks of 5+ ion of bovine insulin recorded with microspray (flow-rate 0.5 μl min^{-1}) under different conditions. The top spectrum was recorded using full sensitivity of the instrument. As a result, the count rate measured in this group of isotopic peaks was approaching one ion TOF pulse per TDC dead-time (20 ns) for a single anode, and the isotopic pattern shows definite signs of saturation, similar to those in Fig. 2. In an attempt to reduce the ion current and avoid saturation, the detector voltage was reduced from 2400 to 2130 V, and the resulting spectrum is shown in Plate 1B. The count rate was in fact suppressed by a factor of ~20; in addition,

isotopic peaks became sharper and the resolution seemed to increase substantially, from ~15 000 to >22 000. The only fact spoiling this idealistic picture is that all isotopic ratios are significantly 'exaggerated' in this spectrum, and the smallest isotopic peaks have almost disappeared. However, all these changes can be explained perfectly well if one considers the mechanism of the ion detection. Lowering the detector voltage by 270 V means that the ion pulse amplitude measured after the MCP plates is reduced by a factor of five or more; almost no single ions can be recorded under these conditions because the pulses produced by them are lower than the discriminator threshold. Owing to the high count rate (real, not measured!), the probability of two ions striking the detector simultaneously (within 1–2 ns) is high, and those coinciding ions have a higher chance of overcoming the threshold of the discriminator because their pulses are superimposed. Since the probability of double coincidences is equal to the probability of single events squared, the resulting spectrum better represents intensities squared rather than just intensities reduced by a factor of 20. This transformation of spectra leads to exaggerated peak ratios (not necessarily isotopic) and peak sharpening observed in Plate 1B. However, the latter does not result in the enhancement of the real resolution, because those peaks that were not resolved in a normal spectrum cannot become resolved after all intensities are squared.

Unfortunately, many other methods of ion current reduction also lead to a variety of spectral distortions. Throttling the ion beam by creating small potential barriers may cause time-dependent behavior as a result of space charge accumulation. Defocusing or steering the beam off the axis at the TOF entrance may spoil the mass resolution or at least require recalibration. Moving the electrospray needle further from the sampling orifice seems to be the least harmful way of reducing the ion current, provided that spraying is performed in a clean atmosphere with a low concentration of impurities that could cause proton-transfer reactions. The spectrum shown in Plate 1C was recorded under the same conditions as in (A), but with two exceptions: the sample was diluted to one-third of its initial concentration and the sprayer was moved ~20 mm away from the orifice with respect to its previous position (6 mm to orifice). In contrast to (A) or (B), this spectrum shows no signs of peak saturation or isotopic distortion.

The same spectrum in Plate 1C illustrates also how the four-anode detector may help to enhance resolution. Normally the high resolution of TOF does not compromise its sensitivity, and $R_{FWHM} \approx 10\,000$ can be obtained together with maximum ion transmission. In some cases, sacrificing part of the ion beam may lead to higher resolution values, as illustrated by Plate 1C where a resolution of 15 000 was achieved. This spectrum of bovine insulin ($M_r \approx 5730$ Da) was recorded with one of four anodes of the detector, thus recording about a quarter of the total ion current. Although the multiple-anode detector was not designed for this purpose, using selected 'sweet' anodes may reduce the effect of minor mechanical inaccuracies on resolution. This is somewhat similar to closing a slit in sector instruments, thus reducing the effect of ion-optical aberrations.

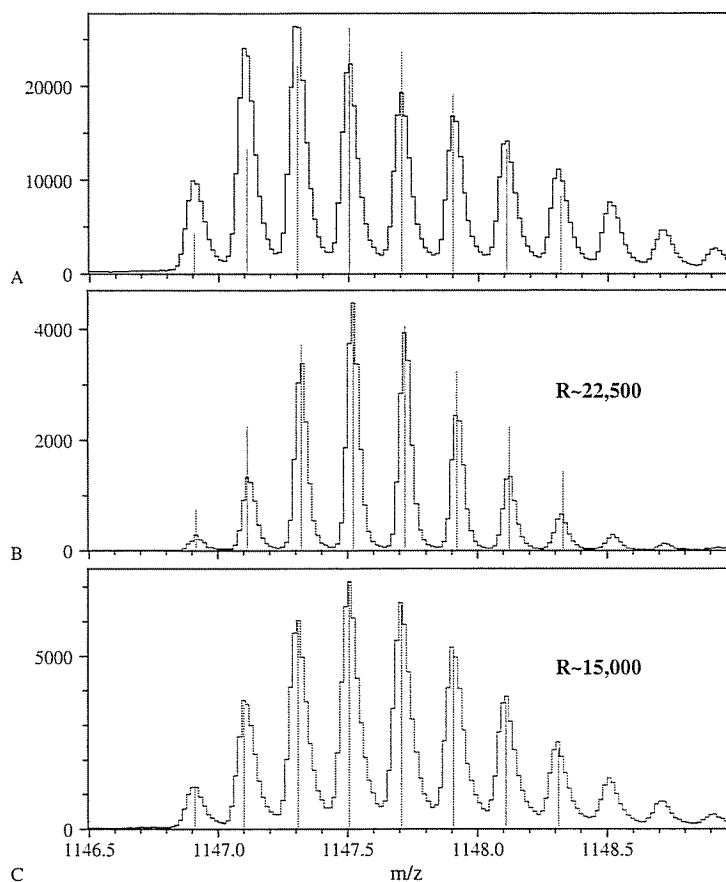**B 8**

Introduction to quadrupole–TOFMS

Plate 1. A segment of the ESI spectrum of bovine insulin. (A) 3 pmol μl^{-1}, sprayer-to-orifice distance 6 mm, MCP detector voltage 2400 V; (B) 3 pmol μl^{-1}, sprayer-to-orifice distance 6 mm, MCP detector voltage 2130 V; (C) 1 pmol μl^{-1}, sprayer-to-orifice distance 20 mm, MCP detector voltage 2400 V. Vertical bars in each spectrum represent the theoretical isotopic distribution.
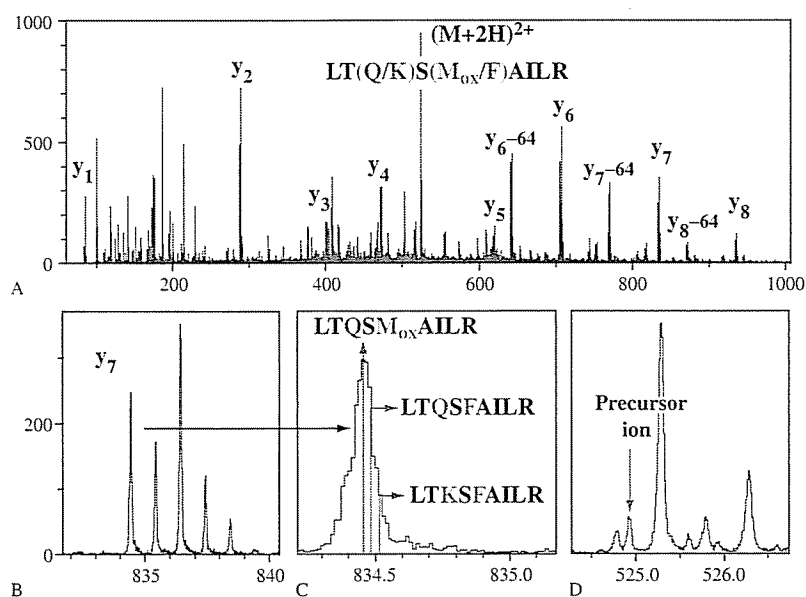
Plate 2. MS/MS of m/z 524.8 (charge 2+) from the tryptic digest of a gel-separated protein. A 3 Th m/z window was selected by Q1 to transmit full isotopic pattern. (A) Full spectrum with the Y series labeled; (B) a segment showing the ^{18}O-labeled Y_7 fragment ion; (C) further expansion of the Y_7 monoisotopic peak with arrows indicating m/z values of the three possible structures on the horizontal axis; (D) the remainder of precursor ions with a singly charged interfering impurity.

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MS/MS OPERATION

The simplest MS/MS operational mode is the so-called 'product ion scan,' where all fragment ions of a particular precursor ion are recorded, thus providing structural information. Since both single and tandem mass spectra are obtained in the TOF analyzer, product ion spectra are recorded with the same high resolution, mass accuracy and even mass calibration as in single MS mode. One of the most useful features of TOF spectra for structural identification is very high mass accuracy, in the ppm range. Many papers have been published recently on this theme,[26-28] especially in the area of protein analysis,[29] and here is just another example. A peptide was sequenced from a ladder of ^{18}O-labeled Y fragments (Plate 2), but contained two ambiguities: two pairs of possible amino acid residues with the same nominal mass, glutamine or lysine (Q or K, $\Delta m = 0.036$ Da), and phenylalanine or oxidized methionine (F or M_{ox}, $\Delta m = 0.033$ Da). The 'smaller' Y-fragment ions (Y_1–Y_4) do not suffer from these uncertainties, so the quality of calibration can be verified on those peaks [e.g. Y_4 in Plate 2(A)], or a spectrum may even be recalibrated (not necessary in this case). Going then through all other Y-fragment ions one by one, and finding the 'best fit' for each of them results in unique sequence LTQSM$_{ox}$AILR [see Y_7 in Plate 2(C)], except for I/L uncertainty. The presence of M_{ox} is confirmed in addition by a series of $Y - 64$ fragments corresponding to a loss of an SCH$_4$O—group.

Although in the above example smaller Y-fragment ions were proposed for recalibration, one should be cautious when performing the lock-mass (single-point) recalibration of product ion spectra. Unless performed on a reliable peak with a single component (no overlaps), internal recalibration may in fact make things worse. In peptide identification studies, there are two popular choices for an internal calibrant: immonium fragment ions or surviving precursor ions, and neither of these choices is perfect. Peaks of immonium ions reside in a densely populated part of spectra, where an overlap with other fragments is highly possible. In addition, if recalibration introduces some error at a lock-mass of ~100 Da, errors for heavier fragments will increase linearly with mass. On the other hand, what is thought to be surviving precursor ions, may in fact be an impurity that accidentally coincides in m/z with the precursor ion of interest. When working with nanospray and low sample concentrations, peaks from singly charged impurities and clusters dominate the spectra; they are present at every nominal mass and may survive CID better than multiply charged peptide ions [see Plate 2(D)] for an expansion of the surviving precursor]. As a result, the candidate for lock-mass calibration may have an m/z value slightly different from what it had in the single mass spectrum.

In MS/MS experiments, the ion count rates are typically several orders of magnitude lower than in single MS, and peak saturation is usually not a problem; on the contrary, every recorded ion may be important when working with low sample concentrations. This is illustrated by Fig. 3, which shows a product ion spectrum of a precursor with m/z 461.3 recorded in 1 s from a low concentration tryptic digest of bovine serum albumin. The whole Y-fragment series is clearly visible there, although the Y_7 peak has an
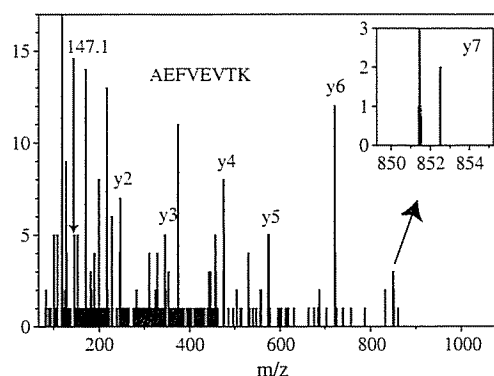

Figure 3. MS/MS of m/z 461.3 (charge 2+) from the tryptic digest of bovine serum albumin. Recorded in 1 s from a 0.1 pmol μl^{-1} solution with nanospray. Inset: expanded view of the Y_7 fragment ion.

amplitude of 3 ion counts only (inset in Fig. 3). In triple quadrupoles, such a peak would be either lost in the noise or ignored owing to poor statistics. In QqTOF instruments, however, it may be considered as a good confirmation of the Y-series extension. This is because of two benefits of TOF technology: high resolution, which results in grouping of counts into narrow (~0.1 Th wide) peaks, and low electronic noise, typically 1–3 counts s^{-1} that are spread across the whole mass range due to parallel ion detection. There were only seven ion counts recorded in the monoisotopic peak of the Y_7 fragment, but the presence of another peak having 2 counts and located almost exactly 1 Th apart (in fact, 1.06 Th) provides enough confidence to treat this peak as a reliable contribution to sequencing data. In a similar fashion, the peak at m/z 147.1, 5 counts high and containing 12 counts only, may serve as a strong indication that this tryptic peptide has lysine at the C-terminus; no counts were registered at m/z 175.1 corresponding to arginine. QqTOF's tolerance to 'poor' statistics becomes especially useful in LC/MS applications where a single spectrum is usually acquired in about 1 s.

PRECURSOR ION SCANS

Since the QqTOF is seen as a replacement for triple quadrupoles in certain applications, it is natural to transfer useful experience and methods from one instrument to another. In triple quadrupoles, precursor ion scans became popular as a tool for identification of the components of a mixture sharing a common structural motif. The loss of a particular diagnostic fragment has been used for identification of phospho- and glycopeptides,[30] peptides,[31] proteins and lipids.[32] However, a strong limitation of the QqTOF instruments is their lower sensitivity in this particular mode of operation compared with triple quadrupoles. The last mass analyzer (TOF or Q3) does not need to record a full spectrum in this mode, and the QqTOF does not benefit from simultaneous ion detection in TOF. On the other hand, more ions are lost in the TOF compared with a third quadrupole: at the TOF entrance, on grids, at the detector and due to low duty cycle. The overall transmission may be of the order of

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1%. A significant part of these losses (factor of 5–20) is due to the low duty cycle of the orthogonal TOF, as discussed above.

To eliminate these losses, ions can be trapped in the collision cell Q2, and then released in a short burst into the TOF modulator and finally to the TOF itself (see Fig. 4). However, being accelerated to the same energy, ions with different m/z acquire different velocities in the gap between the exit lens of Q2 and the TOF modulator. Since the length of this gap L is usually larger than the size of the TOF entrance window l, limited TOF separation of ions occurs in this region. Therefore, this part of the instrument can be viewed as two orthogonally arranged TOF mass analyzers, the first of which is coarse and short (although its function as a TOF analyzer is an unintentional and in some sense undesirable aspect of the design). To improve the duty cycle
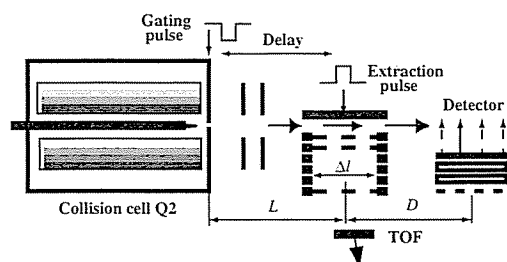
for a particular ion, the release time has to be adjusted with respect to the TOF extraction pulses so that the ions of interest appear in front of the TOF entrance window right at the moment of the extraction pulses. Since the burst of ions can be made short enough, it will easily fit through all restricting windows of the TOF, and a 100% duty cycle will be achieved for ions with preselected m/z.[14]

Since the trapping method has been fully described elsewhere,[14] only a single example is given here. One of the areas where the precursor ion scan mode can provide important information is selective detection of various classes of lipids.[32] Efficiency of detection may be very high for lipids because the head group is often the main charged (positive or negative) product of fragmentation. Phosphatidylcholines and ceramides fall into this category, with the characteristic fragments of m/z 184.074 and 264.15 respectively in positive ion mode. Figure 5 shows spectra of a mixture of phosphatidylcholines and ceramides obtained with nanospray in the normal TOF mode (A) and in the precursor ion scan mode (B, C) with trapping/gating 'on.' Compared with the TOF spectrum, chemical noise is reduced drastically in the precursor ion scan spectrum, as is clear from comparing parts (A) and (B, C) of Fig. 5, or expanded segments of the same spectra shown as insets.

Figure 5 shows that a 1 min scan over a relatively wide mass range still provides high-quality information which demonstrates unit mass resolution along with useful isotopic information. The peak intensity is relatively small (below 200 counts maximum peak height for both spectra), but is

Figure 4. Expanded view of the middle section of the QqTOF instrument illustrating the duty cycle enhancement by means of pulsing ions from the collision cell.

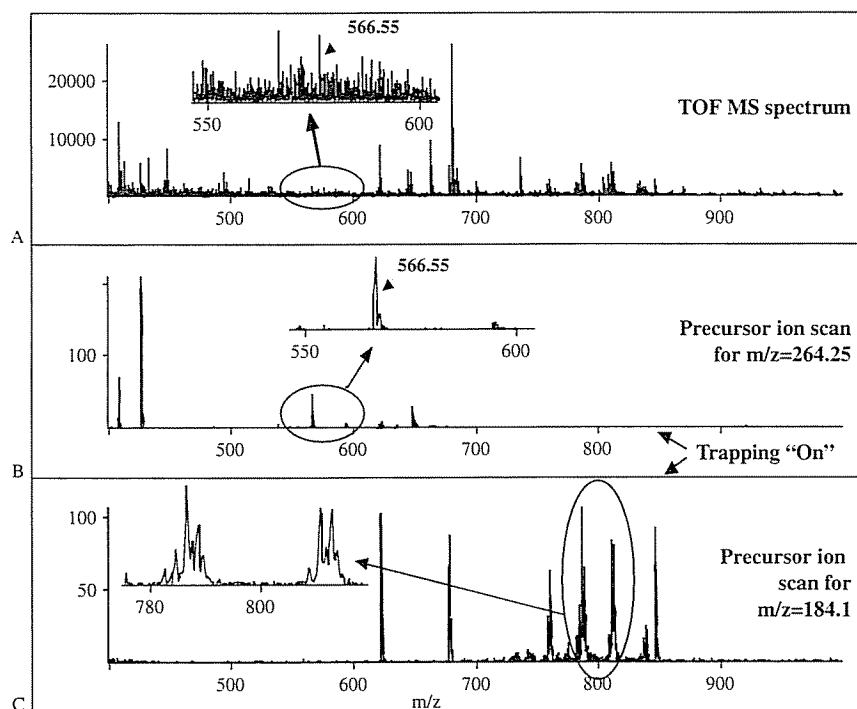**Figure 5.** Nanospray mass spectra recorded from a mixture of phosphatidylcholines and ceramides. (A) Normal TOF spectrum; (B) precursor ion scan for m/z 184.1 ± 0.1; (C) precursor ion scan for m/z 264.25 ± 0.1; the precursor scans were recorded with m/z step size 0.2 Th, dwell time 20 ms and TOF repetition frequency 15 kHz. Collision energy was scanned from 40 to 70 eV from m/z 400 to 1000.

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certainly good enough to indicate the presence of several components of each class in the mixture. Scan speeds which are compatible with chromatography are often possible, using if necessary a reduced Q1 resolution to improve the ion statistics, while still retaining enough mass accuracy to identify target ions for product ion scans. Because of the high specificity and low background noise level, a signal of only a few ions is required in order to provide reasonable confidence in the presence of a precursor ion of interest.

Figure 5 also demonstrates a particular advantage of using a QqTOF instrument rather than a triple quadrupole. Precursor ion scans for multiple product ions can be done in one experiment without loss of sensitivity or speed since, in principle, all products of all precursor ions can be recorded during each precursor ion scan. Thus two or more compound classes, or specific fragmentation paths, can be identified in one experiment. In practice, the trapping conditions must be selected in order to enhance all products of interest, and this can only be done without compromise if the product ions are close together (since the trapping conditions are mass dependent, as discussed previously). In this example, the gating conditions were adjusted (as a compromise) to obtain an approximately equal gain of ~5× for both m/z 184 and m/z 264 fragment ions.

Recently, the same combination of precursor ion scanning and trapping has been successfully applied by Mann and co-workers to detect tyrosine-phosphorylated peptides in complex mixtures.[33]

Although the sensitivity may still be lower than in triple quadrupoles, precursor scans in a QqTOF may benefit from additional features brought by TOF:

1. the above-mentioned possibility of recording several precursor ion scans simultaneously, in a single experiment;
2. the possibility of selecting m/z of the fragment ion with higher resolution, thus increasing selectivity and reducing chemical noise. Note that this can be achieved without additional transmission losses associated with increasing resolution of Q3 in triple quadrupoles.

The described method can be equally well applied to the multiple reaction monitoring (MRM) mode or neutral loss scan mode. Application of the technique to these scan modes would require scanning or stepping the timing parameters with the m/z value of Q1.

Recently, a novel 'precursor ion discovery' method[34] has been described as an alternative to precursor ion scanning for LC/MS applications. During the HPLC gradient the instrument is operated in the single MS mode and switched alternatively at 1 s intervals between low and high collision energy in the collision cell Q2. The first data set at low energy (4 eV) shows all molecular (precursor) ions corresponding to a given retention time, while the second set contains their fragment ions. Whenever a fragment ion of interest occurs in the high-energy data, all its possible precursors are present in the corresponding low-energy data. The mass spectrometer may then switch to the MS/MS mode selecting the potential precursors sequentially to reveal the true parent.

Compared with the true precursor scanning mode, which 'analyzes' precursors in series, choosing them one by one in Q1, the 'precursor ion discovery' method relies on chromatography for crude separation, and then deals with a simplified mixture of precursor ions in parallel. The two methods can be also compared in terms of duty cycle: true precursor scanning with trapping restores the TOF duty cycle, while the 'discovery' method restores the Q1 duty cycle by eliminating scanning.

Many of the above-mentioned benefits of precursor scanning in QqTOF are equally applicable to the 'discovery method:' fragment ion selection with high accuracy and resolution, the ability to monitor several fragments simultaneously and the possibility of performing neutral loss scans in a similar fashion. The precursor ion discovery method works best when the analyte mixtures are not too complex and can be separated well enough by HPLC; otherwise, a true precursor ion scanning may be necessary.

NON-COVALENT COMPLEXES

In the last decade, electrospray ionization has proved to be mild enough to obtain information on the higher order structure of biomolecules.[35] In order to preserve weak non-covalent interactions between or within molecules, the majority of these studies have to be performed close to physiological pH (almost neutral) and with no organic solvents. Under these conditions, the charge acquired by biomolecules or complexes may be much lower than that in acidic solutions that are normally used in studies with peptides and proteins, resulting in m/z values beyond the range of normal quadrupole instruments. Therefore, the ESI-TOF mass spectrometer with its theoretically unlimited m/z range became an instrument of choice for studying large non-covalent associations.[36,37]

One of the 'text-book' examples of the protein non-covalent complexes is human hemoglobin, which forms a tetramer consisting of two α-chains (M_r 15 126 Da) and two β-chains (M_r 15 867 Da), plus a heme (M_r 616.1 Da) per subunit. A nanospray spectrum of this relatively stable complex is shown in Fig. 6(A). Human hemoglobin (Sigma) was desalted by filtering through an M_r 10 000 cut-off centrifugal filter (Microcon, Millipore) and diluted to a final concentration of tetramer of the order of 10 pmol µl^{-1} in 10 mM ammonium acetate (pH 6.5); 3 µl of solution were loaded into a 'short' nanospray needle (Protana, Denmark). All mass spectrometric conditions were the same as usual, including pressures in the quadrupoles; only the collision energy had to be reduced to 2 eV to avoid disruption of weak non-covalent interactions (since the collision gas is used in both MS and MS/MS experiments). As is clear from Fig. 6(A), the tetrameric complex with charge states 17+ and 18+ dominates in the mass spectrum, with minor amounts of dimer, octamer, monomer and heme also visible. Although the sample was desalted, the peaks of the tetramer are wide (see inset); however, the mass determined from the peak maximum ($M_{exp} = 64\,450$ Da) is within 2 Da of the theoretical value ($M_{theor} = 64\,448$ Da). Smaller peaks on the left side correspond to a loss of a single heme.

Studying complexes on a tandem instrument such as the QqTOF may provide more insight into their structure.
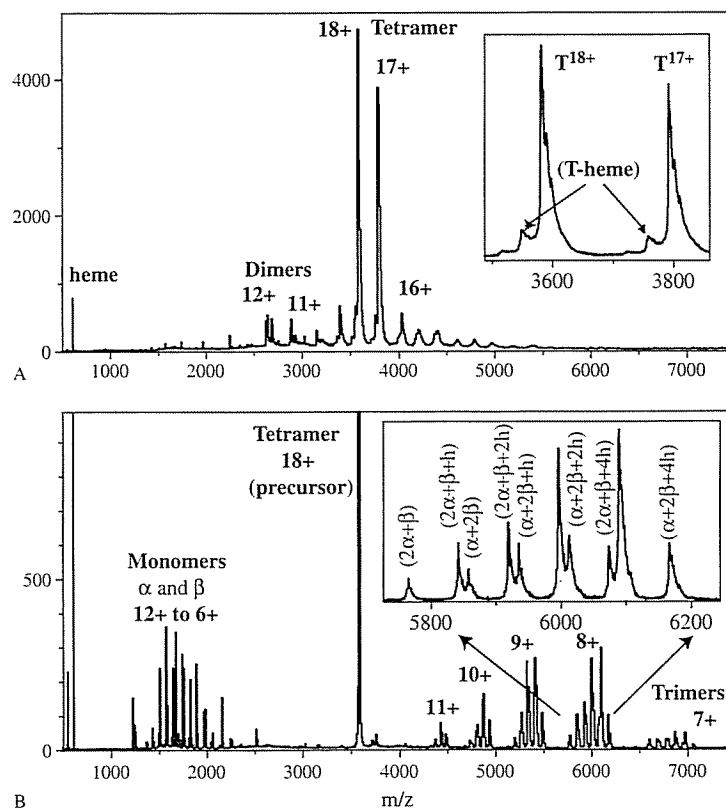
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Figure 6. Nanospray mass spectra of the human hemoglobin tetrameric complex (M_r 64 448 Da). Recorded from a 10 pmol μl^{-1} aqueous solution with 10 mM ammonium acetate (pH 6.5). (A) Single MS; (B) MS/MS of charge state 18+ (m/z 3581) recorded at 20 eV collision energy. Inset: expanded view of 8+ trimers with a different number of hemes.

The tandem mass spectrum obtained by CID of 18+ of the tetramer (m/z 3581) at a collision energy of 20 eV with argon is shown in Fig. 6(B). The majority of tetramers fragment into trimers and monomers, with a surprising distribution of charge: the average charge is almost the same in monomers (9+) as in trimeric fragments. Monomer fragments are found both with and without heme, while trimers appear in all 10 possible ways: ($2\alpha + \beta + n$h) and ($\alpha + 2\beta + n$h), where n is the number of hemes ranging from 0 to 4 (since there were 4 hemes in the original tetramer precursor). Further increase in collision energy results in fragmentation of trimers into monomers, and a loss of heme from both.

ORTHOGONAL MALDI-QqTOF

The TOF analyzer was considered a natural partner for MALDI since the beginning of the method.[38,39] Indeed, MALDI is a pulsed source that is effectively incompatible with scanning mass analyzers such as quadrupoles or sector instruments, which typically require a continuous beam. On the other hand, the laser pulse seems to provide a well-defined start signal for the TOF measurement. However, the match is not perfect. The quality of the pulsed beam is insufficient to obtain the high resolution and mass accuracy necessary for many modern applications. The situation was substantially improved with the introduction of a reflecting TOF scheme[17] and the delayed extraction technique.[40–42]

Delayed extraction partially decouples ion production from the TOF measurement, thus improving the pulsed beam definition. However, some problems still remain. Among them are a complicated calibration procedure, which has to take into account laser power, matrix used and delayed extraction parameters, high ion production for each laser shot, which brings space charge and detector saturation problems, the necessity for fuzzy logic algorithms for the laser fluence control, etc.

Many of the above problems are resolved when an even higher degree of decoupling of the MALDI ion production from the mass measurement is used.[43,44] The technique is called orthogonal MALDI (oMALDI)-TOF with collisional cooling. Here, the pulsed ion beam is converted into a quasi-continuous beam in a collisional focusing ion guide and then introduced into an orthogonal TOF instrument in the same manner as described above for electrosprayed ions (shown in Fig. 7). The pulsed MALDI ion beam spreads in time as it passes through the collisional ion guide. In MALDI, ions are typically generated by a few nanosecond wide laser pulse. As the ions leave the collisional ion guide, the width of their temporal distribution increases up to tens of milliseconds.[43] This represents a time spread of over six orders of magnitude! For comparison, ions from the previous laser pulse will be still leaving the ion guide when the ions from the next pulse start to arrive at a pulse repetition rate of tens of pulses per second. Under these conditions, the beam coming out of the

ion guide may be considered quasi-continuous, becoming almost continuous at higher laser repetition rates.

In addition, the collisional ion guide improves the quality of the ion beam, as outlined above. Thus, the quasi-continuous MALDI ion beam and the continuous ESI ion beam feature similar space and velocity distributions at the exit of the collisional ion guide. Owing to this similarity, MALDI mass spectra inherit most of the beneficial characteristics of the ESI mass spectra in orthogonal TOF, such as high resolution throughout the entire spectrum and high mass accuracy.

Some extra advantages of MALDI with collisional cooling arise from the fact that the ions 'forget' their initial conditions in the ionization region as they move along the ion guide and experience a large number of collisions. Because of that, target/spot irregularities, higher than usual fluence and target surface charging have no effect on resolution and mass accuracy in the orthogonal TOF section. This is dramatically different from the conventional MALDI-TOF where the above factors represent an important problem.

The only drawback of the oMALDI-TOF with cooling compared with conventional MALDI-TOF is its lower transmission efficiency. Conventional axial TOF can transmit almost 100% of the MALDI ions having a relatively broad mass range (about an order of magnitude). Orthogonal injection TOF has the duty cycle losses described above, limiting transmission efficiency over a broad mass range. However, two extra features of the oMALDI-TOF with cooling can actually compensate for the reduced transmission.

First is the ion counting as an alternative to analog detection. The analog detection system (integrating transient recorder) is required for the axial MALDI-TOF because of the large number of ions (typically 10^4–10^5 per laser shot) that are recorded during a single TOF measurement (TOF extraction pulse). Electronic noise is accumulated in the analog detection mode, making it difficult or impossible to distinguish mass spectral peaks with only a few ions. In the oMALDI-TOF, ions that are generated by one laser shot spread out in time, thus allowing hundreds of the TOF measurements to be carried out as the ions leak out of the ion guide. In this case, tens of ions are normally detected during a single TOF measurement. Thus, a TDC (or better

multichannel TDC) can be used for spectra recording similar to the recording of the ESI spectra in QqTOF. Peaks with only a few ions can be detected since the noise of the MCP detector is usually negligible.

Second, a higher than usual laser fluence can increase the overall sensitivity of the instrument. Indeed, the MALDI ionization efficiency increases steeply with laser fluence above the threshold.[45,46] Users of axial MALDI-TOF are forced to operate at near threshold fluences owing to the problems associated with the large number of ions produced in a single shot. Since the oMALDI-TOF with cooling is capable of handling larger numbers of ions per laser shot, more ions can actually be produced from the same amount of sample. Fluence in the oMALDI is typically limited by the increase in metastable fragmentation. Therefore, 'colder' matrices such as 2,5-dehydroxybenzoic acid (DHB) can be favorable in many cases.

As already mentioned, one of the most important features of oMALDI with collisional cooling is decoupling of the ion production from the TOF measurement. Thus, extra ion manipulation stages can be introduced between the MALDI source and the TOF analyzer. For example, the MALDI source can be installed in front of the QqTOF instrument.[44,47,48] In this case the MALDI-QqTOF will operate in the MS or MS/MS mode similarly to the ESI-QqTOF. That gives the MALDI-QqTOF spectrometer an important advantage over the conventional axial injection MALDI instrument in obtaining structural information by MS/MS measurements. In the latter device such measurements have normally been carried out by the so-called post-source decay (PSD) technique, in which a parent ion is selected by an ion gate in the flight tube, and the products of its metastable decay are observed after reflection.[49,50] However, this method has had limited success because of low resolution on the parent ion selection, difficulties in controlling the fragmentation processes and problems in data handling. On the other hand, MS/MS measurements on MALDI ions in a QqTOF instrument can be carried out with the same high resolution, mass accuracy and even mass calibration as in a single MS mode.

Protein analysis applications, such as protein identification, investigation of mutations and chemical modifications, and protein sequencing are among popular applications where both MS and MS/MS are often important. ESI or MALDI can be used for these purposes, with their own benefits and drawbacks. Advantages of MALDI include higher tolerance to contamination and simpler sample handling.

The lack of good quality MS/MS capabilities in conventional MALDI-TOF have led several groups to a layered approach to protein identification.[51] Small fractions of all samples are first analyzed in the single MS mode using conventional MALDI-TOF, which in about 80% of cases solves the problem (according to one study[48]). MS/MS is required in the remaining 20% of the cases and can be carried out using ESI-QqTOF or tandem instruments of other types.

On the other hand, MALDI-QqTOF allows both MS and MS/MS analysis to be performed from the same sample on the same instrument, thus simplifying the procedure. Moreover, unlike nanospray, MALDI spectral acquisition
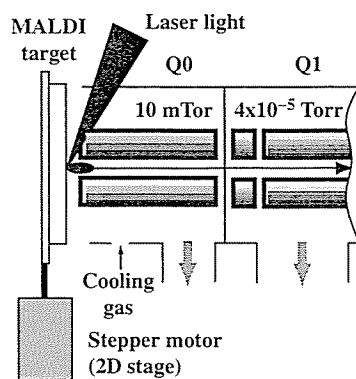

Figure 7. Schematic diagram of the front end of QqTOF with MALDI source.

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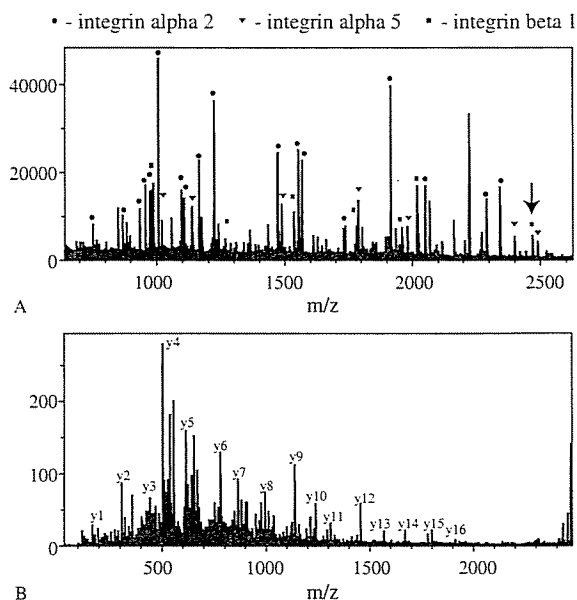862   I. V. Chernushevich, A. Loboda and B. Thomson

Figure 8. MALDI spectra acquired from a tryptic digest of a gel band. (A) Single mass spectrum. Tryptic peptides from three proteins are present, two of which were identified by peptide mapping. (B) Tandem mass spectrum of the peptide marked by the arrow; the third protein (integrin beta 1) was identified using MS-Tag database search.

can be interrupted at any time, and the acquired data can be subjected to analysis. After the analysis, MS/MS of only unidentified peaks can be performed. Such 'smart data acquisition'[52] facilitates efficient use of sample. For example, Fig. 8(A) shows the MALDI spectrum of the tryptic digest of an isolated gel band of human integrin[53] acquired by MALDI-QqTOF in the MS mode. Two components, integrin alpha 2 and integrin alpha 5, were identified using a database search based on peptide mass fingerprinting.[54] Unidentified peaks were subjected to MS/MS and identified using the MS-Tag[55] database search engine. Some of the peaks appeared to be products of non-standard cleavage of integrin alpha 2 or alpha 5, while other peaks revealed the presence of integrin beta 1 in the mixture. Figure 8(B) shows the tandem mass spectrum of the integrin beta 1 digest peptide indicated by the arrow; only Y-fragment ions are labeled.

As illustrated by Fig. 8, MALDI-QqTOF is capable of identifying proteins in samples containing simple mixtures. In most cases when a mixture contains only a few components whose relative abundances differ by a factor of ~10, MALDI-QqTOF can reliably identify all of the components. More complex mixtures may be interrogated by LC/ESI-MS with on-line MS/MS capabilities, as will be described below. Alternatively, the complexity of such mixtures may be reduced by additional off-line separation by other methods, including chromatography and electrophoresis, either before or after digestions. Then, the simplified fractions can be collected onto MALDI target for further analysis.

AUTOMATED DATA ACQUISITION AND PROCESSING

Modern applications of mass spectrometry often demand that samples be processed and analyzed in an automated or semi-automated fashion, either because of the complexity of the sample or the sheer number of samples to be analyzed, or because the data system can perform a more reliable function than an operator who uses less objective rules of interpretation. In the case of a tandem mass spectrometer, qualitative analysis requires that tandem mass spectra be acquired on precursor ions of interest; since the volume of data precludes acquiring tandem mass spectra on every ion in the single mass spectrum, some selection rules must be applied in order to optimize the information content and reduce the volume of data (and hence increase the likelihood of extracting useful information from it). The quality of the spectral information from the TOF system provides several opportunities for making well-educated decisions about which precursor ions to select for MS/MS,[56,57] while parallel ion detection makes possible sub-second spectra acquisition. In a typical scenario, the sample is introduced through an LC column, which allows tandem mass spectra to be acquired on one component at a time (although, in practice, co-eluting species are common, so the system has to be flexible enough to identify and fragment more than one ion during peak elution time).

In the most general case, TOF mass spectra are acquired as the sample elutes from an LC column, and the data system analyzes the mass spectra in real time in order to decide which precursor ion to select for MS/MS. If an ion meets the selection criterion (e.g. intensity, charge state, m/z range), then the system automatically switches to the MS/MS mode (this step generally only requires changing the voltages on elements of the ion optical path, a process which takes only a few milliseconds). After acquiring tandem mass spectra for a fixed time or until meeting certain criteria, the system returns to the MS mode in order to wait for the next component of interest. The system can be directed to continue acquiring TOF mass spectra until another ion exceeds the threshold for MS/MS, or it can be programmed to alternate between MS and MS/MS, selecting the most likely candidates in each TOF mass spectrum.

A simple example of the benefit of this method is shown in Plate 3. This shows a micro-LC/MS/MS analysis of 20 fmol of a tryptic digest. Over the entire run length of 70 min, ~350 TOF mass spectra were acquired, each of which contains many peaks consisting of solvent, impurities and peptide ions. The system was programmed to perform two MS/MS experiments after each TOF mass spectrum, using the most intense peak in the spectrum and performing MS/MS at two different collision energies. This strategy ensures that sufficient fragmentation information will be obtained on any component, without having to predict the optimum energy in advance. Other strategies that can be used are to calculate a collision energy based on the m/z of the precursor. While the TIC of this experiment shows little useful information, the selection of base peaks for MS/MS resulted in the acquisition of high-quality MS/MS data on several peptide ions. Plate 3(C) shows an example of the
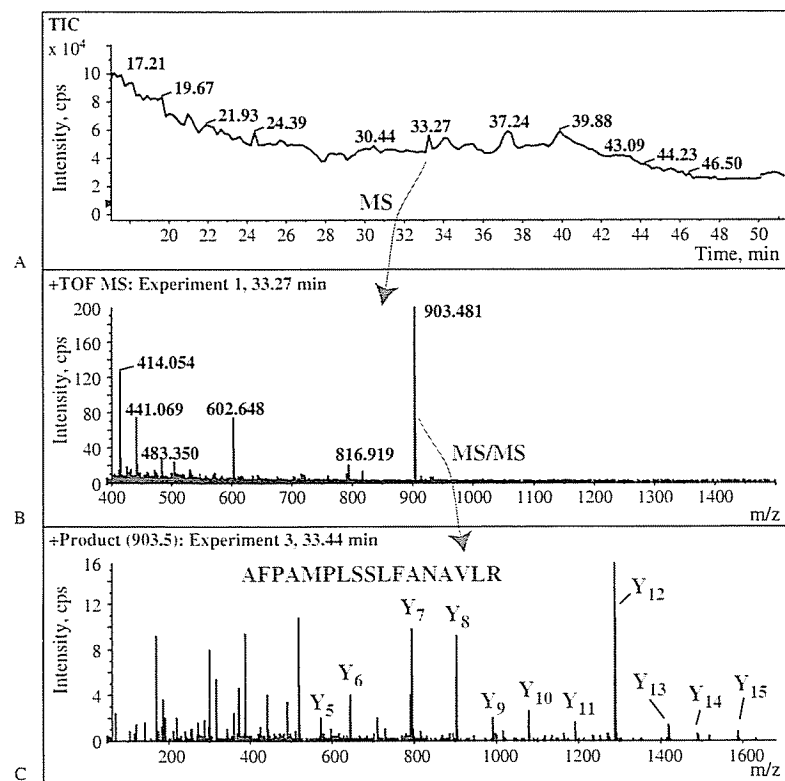
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Plate 3. Information-dependent LC/MS/MS from 20 fmol of a tryptic digest. PepMap column, 75 μm i.d. × 15 cm long (LC Packings). (A) TIC; (B) TOF spectrum recorded at $t = 33.27$ min; (C) MS/MS of a peptide with m/z 903 (2+) (Experiment 2, not shown, was a lower energy MS/MS spectrum).
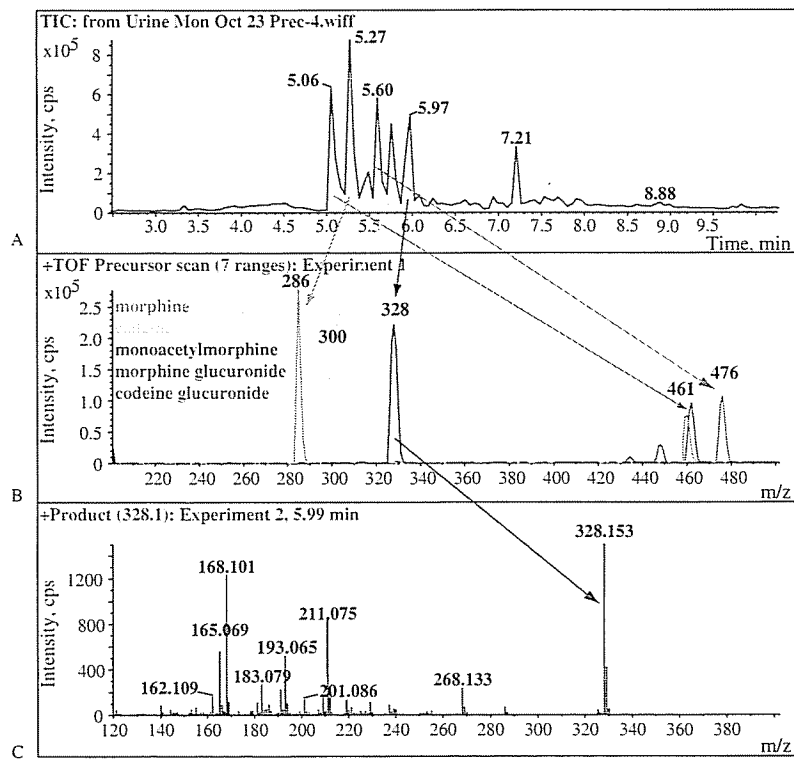

Plate 4. Information-dependent LC/MS/MS with precursor ion scan as a survey scan; 10 μl of a urine sample from a subject exposed to monoacetylmorphine were injected. (A) TIC; (B) overlaid precursor ion scans from five major chromatographic peaks (recorded in 2 s each); (C) MS/MS of monoacetylmorphine recorded in 1 s after detecting m/z 328 in the precursor ion scan.

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MS/MS of m/z 903.5 which occurred at a retention time of 33.27 min, where only a small peak was visible in the TIC. The quality of data was good enough to create a sequence tag and perform the database search.

This approach can accommodate co-eluting components by using a dynamic exclusion window after each tandem mass spectrum, in order to avoid acquiring more tandem mass spectra on the same precursor. In this way, if two peptide ions are present in the TOF mass spectrum, the most intense will be selected for the first experiments, and the less intense will be selected for the second MS/MS experiment (both being acquired at two different collision energies in this case). Typically the exclusion window is applied for the normal width of an LC peak, so that if a later eluting component has the same m/z, it can still be selected for MS/MS.

Other selection rules can also be used. For example, the charge state of the precursor ion can be determined by the isotopic spacing. For peptide ions, it is usually desirable to select the doubly charged or triply charged ions for MS/MS (this also discriminates against singly charged background peaks), and the experiment may be arranged so that only doubly or triply charged ions are selected for MS/MS. Selection rules can also be applied to include a list of suspected or known 'target' ions, which will be selected for MS/MS if they appear during the run below the normal threshold, and also to include a list of excluded ions which will always be ignored no matter how abundant. These rules can be applied with or without time windows associated in order to include known or suspected retention times.

The availability of other scan modes also offers other ways of choosing the precursor ions for MS/MS. To increase specificity of the precursor ion selection for LC/MS/MS experiments, the precursor scan mode can be used to perform the survey scan (instead of TOFMS). Combined with a simple threshold, this can be used to detect and acquire tandem mass spectra of all precursors which form a particular (or more than one particular) product ion. For example, a precursor scan of m/z 86 can be used to detect all peptides that contain leucine/isoleucine; upon detection of even a very weak signal (2–3 counts may be sufficient), the system can stop scanning Q1, and perform a full MS/MS acquisition on the detected precursor, achieving improved S/N compared with acquiring tandem mass spectra on all precursors. Combined with the pulsing function described earlier, which enhances the sensitivity to either a particular product ion, or to the entire product ion range, this can provide a sensitive and specific method of focusing on a compound class of interest. An example below illustrates how this method is applied to studies of drug metabolism. Drug metabolites often have a similar fragmentation pattern, which makes them good candidates for detection through multiple precursor ion scanning. Although this model example deals with well-known metabolites of monoacetylmorphine (MAM), the same method may be applied with certain precautions to newly developed drugs.

In a preliminary study, common fragments of MAM metabolites were determined: product ion spectra were recorded from several metabolites (not shown), and seven fragments with m/z values 153.1, 155.1, 165.1, 181.1, 183.1, 191.1 and 193.1 were selected for multiple precursor ion scanning.

A urine sample from a subject exposed to MAM was analyzed by LC/MS: 10 µl were injected into a 2 × 100 mm C_{18} column (Luna Phenomenex, 3 µm) at a flow-rate of 200 µl min^{-1}. During the LC run, the mass spectrometer was switching between 2 s precursor ion scans for the above-mentioned fragment ions and 1 s acquisition of product ion spectra. Precursor ion scans were performed in the m/z range 200–500 Th with a step of 1 Th and 45 eV collision energy with argon. Whenever the intensity of the largest peak signal exceeded the threshold, the mass spectrometer switched automatically to acquisition of the product ion spectrum of the found precursor. Simultaneously, the collision energy was lowered to 35 eV to preserve some of the precursor ions for more accurate mass determination.

The results are illustrated in Plate 4, where (A) is the TIC obtained from both types of scan (precursor and product ion scans), (B) shows five overlaid precursor ion spectra corresponding to major peaks in the chromatogram and (C) shows a product ion spectrum recorded after detection of MAM (m/z 328) at a retention time of 5.97 min. Since precursor ion scans were recorded with a low resolution (2–3 Th Q1 transmission window) and a coarse step of 1 Th, one cannot expect to obtain in 2 s beautiful spectra with high resolution and high mass accuracy; these spectra serve for detection purposes only. The actual mass is then determined with the ppm accuracy of TOF from the corresponding product ion spectra Plate 4(C). All main metabolites were easily detected in the chromatogram, and five out of six major peaks in TIC correspond to monoacetylmorphine metabolites. The peak eluting at 7.21 min does not belong to the family and is a result of coincidence in m/z of an abundant fragment.

The abundances of seven fragment ions were summed together in the precursor ion scans described above. To increase the specificity even more, precursor scans for separate fragment ions may be treated individually, and software may require the abundances of several fragment ions to be above the threshold before the instrument is switched to the product ion scan mode. In this case TIC would still contain a peak at $t = 7.21$ min, but the MS/MS experiment would not be performed.

In a similar fashion, the precursor ion discovery method or neutral loss discovery method[58] can be used to identify and select target precursor ions for MS/MS. Recently, it was shown how a combination of isotopic pattern (peaks separated by 8 Da using the ICAT labeling method) and relative abundance could be used to very specifically identify target precursors, requiring that the abundances of the discovered ions be sufficiently different to indicate a significant difference between control and modified protein samples.[59]

In general, then, the data systems can acquire and process single mass spectra in real time, make a decision based on one or more rules and acquire one or more tandem mass spectra on a time-scale which is fast enough to be useful chromatography. The quality of the decision, however, and therefore the quality of the data, still depend on the

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original quality of the TOF mass spectrum—signal-to-noise ratio, counting statistics, mass resolution and accuracy—and also on the ability of the computer to process data in real time (peak finding and centroiding, de-isotoping, smoothing, deconvoluting multiply charged spectra, integrating peak areas). The high sensitivity, resolution and mass accuracy of the QqTOF mass spectrometer combined with parallel ion detection make it possible to develop a wide variety of methods that would suit different needs. The fantasy of a researcher is limited in this case mostly by the available software.

CONCLUSION

We have shown how significant advances in the development of quadrupole time-of-flight instrumentation have been made in a relatively few number of years, and the story is still far from complete. While originally seen as a qualitative technique, able to provide high mass accuracy and sensitivity in a product ion mode with electrospray ionization, recent events have seen the extension to MALDI, to other scan modes conventionally associated with triple quadrupole instruments and to new heights of mass resolution and sensitivity. It seems probable that these and other developments to come will carry the technique into new application areas, and into areas of application traditionally associated with other mass spectrometer systems. Barriers which seemed significant a few years ago, associated with speed of acquisition, large volumes of data and complexity of operation, are falling rapidly to advances in raw computing power and sophisticated data processing. The cost of instrumentation has decreased even as performance has increased (a signature of the entire field of mass spectrometry). While the quadrupole time-of-flight combination is not likely to dominate the field of mass spectrometry completely, some of the limitations of the technique which appear significant today will likely be overcome by further inventiveness, leading to even wider use in both qualitative and quantitative applications in the future.

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